IDENTIFICATION OF REAGENTS FOR THE DIAGNOSIS AND STUDY OF FRANCISELLA

FIELD

The present disclosure relates to the analysis of bacteria. Specifically, the current disclosure relates to reagents that are capable of identifying bacteria causing human disease and/or posing a biological threat.

BACKGROUND

The growing concern for the use of biological agents as weapons in terrorist incidents has prompted Federal health agencies to accelerate measures to protect the public from such threats. In February, 2002, the National Institute of Allergy and Infectious Diseases (NIAID) released its biodefense research agenda for CDC category A agents. The agenda described the high priority goals for immediate, intermediate and long-term research related to counter bioterrorism (1).

Francisella tularensis, the agent causing tularemia, is a CDC category A agent and is considered as a major biological threat. *F. tularensis* has long been considered as a potential biological weapon. In the 1950's and the 1960's, the US military developed weapons that would disseminate *F. tularensis* aerosols and by the late 1960s, *F. tularensis* was one of several biological weapons stockpiled (2). A parallel effort by Soviet Union continued into the early 1990s and resulted in weapons production of *F. tularensis* strains engineered to be resistant to antibiotics and vaccines (3). *F. tularensis* is a good candidate for a biological weapon due to its very high infectivity. The infectious dose can be as low as 10 to 50 microorganisms if inhaled (4). In 1969, a World Health Organization expert committee estimated that an aerosol dispersal of 50 kg of virulent *F. tularensis* over a metropolitan area with 5 million inhabitants would result in 250,000 incapacitated casualties, including 19,000 deaths (5). Recently, the Centers for Disease Control and Prevention examined the expected economic impact of bioterrorist attacks and estimated that the total base cost to society of an *F. tularensis* aerosol attack would be $5.4 billion for every 100,000 persons exposed (6).

Tularemia is a zoonotic disease caused by *F. tularensis* and occurs mainly in the Northern Hemisphere (reviewed in 7). In the United States, the disease is most prevalent in Arkansas, Illinois, Missouri, Texas, Virginia and Tennessee (8). Tularemia agent is primarily recovered from lagomorphs (rabbits), rodents and arthropods (ticks and deer flies) within the United States

and from water, mosquitoes and aquatic mammals outside the United States. The rabbit is the vertebrate most commonly associated with tularemia in North America. The reported incidence of tularemia in the United States since 1967 has been fewer than 200 cases per year (9). However, in the event of an intentional release, a large number of cases in a local geographical area would be expected.

Tularemia is acquired under natural conditions by direct inoculation (such as, but not limited to, arthropod bite), animal contact such as, but not limited to, skinning or eating infected animals, by drinking contaminated water, or via the airborne route (10-12). As few as 10 to 50 microorganisms given by aerosol can cause infection in humans (4). To date, tularemia transmission from patient to patient has never been reported.

Tularemia can be divided into the ulceroglandular (~75% of the patients) and the typhoidal (~25% of the patients) forms, based on the clinical symptoms. The average incubation period is 3 to 6 days. Patients with the ulceroglandular form of the disease develop nonspecific symptoms consisting of fever, chill, headache cough and myalgia (13). A cutaneous ulcer occurs, associated with large peripheral lymph nodes (> 1cm) in 60% of patients and is the most common sign of tularemia. The typhoidal form presents symptoms very similar to the ulceroglandular form, but lymph nodes are usually smaller (< 1cm) and skin lesions are generally absent. Approximately 80% of the patients with the typhoidal form also contract pneumonia; this linkage probably accounts for the higher mortality associated with this form of the disease (30-60% if untreated) (8). As a result of its higher mortality, the typhoidal form would be the most likely form in case of an act of bioterrorism.

A live attenuated vaccine has been developed from the avirulent live vaccine strain (LVS) initially used by the Russian in the 1930's (14, 15). It is used to protect people routinely working with *F. tularensis*. This vaccine has been shown to protect human volunteers against an aerosol challenge with virulent *F. tularensis* (16). Recent investigations of mouse models have suggested that the delivery site may influence the host defense mechanisms employed to combat the infection (17). The LVS is currently available as an Investigational New Drug and is under review by the US Food and Drug Administration.

Rapid diagnostic testing for tularemia is not widely available (reviewed in 41, 42). The organism can be isolated from blood, sputum, skin, or mucosal membrane lesions of an affected individual, but microbiologic diagnosis can be difficult due to unusual growth requirements for *Francisella* strains and/or due to the overgrowth of commensal bacteria. Diagnosis of primary typhoidal tularemia is also difficult because signs and symptoms are nonspecific and frequently there is no suggestive exposure history (43). Because of its infrequent occurrence, its symptoms

might be misidentified, resulting in a delay in starting effective antibiotic treatment. In the case of an intentional or accidental release of aerosolized *Francisella* strains, such as, but not limited to, *F. tularensis*, it may be the clustered occurrence and unusual epidemiologic features that alert authorities to the intentional release. A number of assays may be used to identify *Francisella* strains, such as, but not limited to, *F. tularensis*, but the availability of such assays (for example, antigen detection, PCR, ELISA; compared in (44)) may only be available in research and reference laboratories. Serologic assays to detect the presence of specific antibody may not be diagnostic for many days after infection and would hinder effective treatment and/or containment of an intentional release. Any diagnostic reagent for strains of *Francisella* would be a major benefit, since the treatment for tularemia is the same for all strains (62-64). In cases of geographically concentrated outbreaks of tularemia (such as, but not limited to in a biological attack, an inadvertent release or a naturally occurring outbreak), the inability to rapidly diagnose the causative *Francisella* agent could be the key impediment to an effective public health response.

Therefore, a rapid and sensitive diagnostic assay is needed to allow a rapid response in the event of an intentional or accidental release of pathogenic *Francisella* strains. Such a diagnostic assay is currently lacking in the art.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows a representative diagram of the Negative/Positive biopanning procedure used in the present disclosure. In this example, peptide sequences that bind to *F. tularensis* LVS and not to *F. philomiragia* were isolated using both negative and positive selection steps as indicated (59). Phage that did not bind *F. philomiragia* was applied to *F. tularensis* LVS and the bound phage were eluted with low pH buffer. The eluted phages were then amplified in additional rounds of selection.

FIG. 2 shows relative phage binding to *F. tularensis* LVS, *F. novicida* and *F. philomiragia*. 4-4-2 phage (expressing peptides having the sequence of TSITPWFFLSRP) (SEQ ID NO: 2) and OV1 phage (negative control, expressing peptides having the sequence of TGRHTPWPHFMP) (SEQ ID NO: 4) were used. Identical aliquots of phage were allowed to bind for 2 hours at each temperature shown. The columns show the ratio of binding to *F. tularensis* LVS and *F. novicida*, compared to *F. philomiragia* (ratio = 1). The ratios observed did not significantly change when the amount of phage added was varied from 0.1 to 100 phage added per bacterium (data not shown).

FIG. 3 shows phage binding to *F. tularensis* LVS (FT), *F. novicida* (FN), *F. philomiragia* (FP), *S. aureus* (SA), *E. coli* (EC), and *E. hirae* (ENT) at 4°C and at 37°C. 4-4-2 = 4-4-2 phage expressing peptides having the sequence of TSITPWFFLSRP) (SEQ ID NO: 2); negative = OV1 phage (expressing peptides having the sequence TGRHTPWPHFMP) (SEQ ID NO: 4).

FIG. 4 shows Trypsin sensitivity of phage binding to *F. tularensis* LVS (FT) and to *F. philomiragia* (FP) at 37°C. 4-4-2 = 4-4-2 phage expressing peptides having the sequence of TSITPWFFLSRP) (SEQ ID NO: 2); negative = OV1 phage (expressing peptides having the sequence TGRHTPWPHFMP) (SEQ ID NO: 4).

FIG. 5 shows the specificity of phage binding to *F. tularensis* LVS and *F. novicida*. 1×10^{11} bacteriophage were mixed with peptide p442 or p442S at the concentration shown and allowed to bind to 1×10^9 *F. tularensis* LVS or 1×10^9 *F. novicida* bacteria for 1 hour at 4°C in 1x PBS. After binding, bacteria were washed three times with ice cold 1 x PBS. Bound phage were eluted with 0.2 M glycine buffer (pH 2.2) and titered on *E. coli* strain ER2738. Phage used for binding were 4-4-2 (expressing peptides having the sequence of TSITPWFFLSRP) (SEQ ID NO: 2) or OV1 (expressing peptides having the sequence TGRHTPWPHFMP) (SEQ ID NO: 4) which served as a negative control. p442 = competing peptide with sequence TSITPWFFLSRP (SEQ ID NO: 2); p442S = non-competing peptide with the sequence SLFWTTPIPSFR (SEQ ID NO: 5).

FIG. 6 shows the effect of buffer composition on phage binding to *F. tularensis* LVS (FT), *F. novicida* (FN) and *F. philomiragia* (FP). 10^{11} bacteriophage were added to 10^9 bacteria in 0.2 ml of the Tris-Mg buffer, 1X PBS and 0.1X PBS as indicated. After 1 hour at 4°C, the bacteria were washed 3 times with the same buffer used in the binding reaction (4°C). Bound bacteriophage were eluted with 0.2 M glycine (pH 2.2) and titered on *E. coli* strain ER2738. Phage used for binding were 4-4-2 (expressing peptides having the sequence of TSITPWFFLSRP) (SEQ ID NO: 2) or OV1 (expressing peptides having the sequence TGRHTPWPHFMP) (SEQ ID NO: 4) which served as a negative control.

FIG. 7 shows the effect of growth media on phage binding to *F. tularensis* LVS (FT), *F. novicida* (FN) and *F. philomiragia* (FP). Bacteria were grown in either trypticase soy (TSB) or Chamberlain minimal medium to mid-log phase. Bacteria were collected by centrifugation and resuspended in 1X PBS at a concentration of 5×10^9 bacteria/ml. 10^{11} bacteriophage were added to the bacteria in 0.2 ml of 1X PBS. After 1 hour at 4°C, the bacteria were washed 3 times with 1X PBS (4°C).

Bound bacteriophage were eluted with 0.2 M glycine (pH 2.2) and titered on *E. coli* strain ER2738. Phage used for binding were 4-4-2 (expressing peptides having the sequence of TSITPWFFLSRP) (SEQ ID NO: 2) or OV1 (expressing peptides having the sequence TGRHTPWPHFMP) (SEQ ID NO: 4) which served as a negative control.

DETAILED DESCRIPTION Study of the biology and the mechanisms of pathogenicity of *Francisella* have been impaired by a lack of research and diagnostic tools. This lack of information has hampered the development of diagnostic assays and kits, as well as the identification of novel proteins and other targets for use in drug discovery programs and in vaccines. Therefore, it would be advantageous to provide reagents that identify *Francisella* strains, such as, but not limited to, those identified in playing a role in human diseases or those identified as capable of being used as a biological weapon. In one embodiment peptide sequences are identified as such reagents. Furthermore, it would be advantageous to provide diagnostic assays using the reagents identified for the identification of *Francisella* strains, such as, but not limited to, -those identified in playing a role in human diseases or those identified as capable of being used as a biological weapon. Further still, it would be advantageous to provide diagnostic assays suitable for the rapid detection of such *Francisella* strains. Such rapid detection may prove useful, for example, in the event of an intentional or accidental release of *Francisella* strains. Finally, it would be advantageous to provide such reagents as research tools for the study and characterization and to provide vaccines useful for protection against *Francisella* strains those identified in playing a role in human diseases or those identified as capable of being used as a biological weapon.

Francisella strains, such as *F. tularensis*, are tiny, non-motile, aerobic, gram-negative coccobacillus. These strains are fastidious organism and usually requires cysteine supplementation for good growth on general laboratory media. *F. tularensis* has a thin capsule consisting of lipids, carbohydrates and proteins, all of which may contribute to the virulence (18). Biochemically, the organism is fairly inert, using glucose as the carbon source. It is a hardy non-spore forming microorganism, that is able to survive for weeks at low temperatures in water, moist soil, hay and decaying animal carcasses (19). *F. tularensis* has been divided into two major biovars (20). *F. tularensis* biovar *tularensis* (type A) is the most common in the United States and is recovered from rodent and ticks and is highly virulent for rabbits and human. *F. tularensis* biovar *palearctica* (type B) is more common outside the United States and is recovered from water, mosquitoes, and aquatic mammals, and is relatively avirulent for rabbits and human. Both subspecies cannot be distinguished serologically although they may be differentiated by 16S RNA sequencing (21). Other *Francisella* strains include *F. philomiragia* (which shares a moderate 30-

40% homology to *F. tularensis*) and *F. novicida* (which shares an approximate 90% homology to *F. tularensis*) (22).

Very little is known about the mechanisms of virulence of *Francisella*. This pathogen is capable of growing in macrophages from rodents including mice, rats and guinea-pigs as well as human monocytes (23, 24). *Francisella* appears to be able to inhibit phagosome-lysosome fusion (23). However, the intracellular compartment occupied by *Francisella* is acidified, an event thought to facilitate acquisition of iron, which is essential for growth (25). There is also evidence that *Francisella* can replicate in some types of phagocytic cells, such as, but not limited to, fibroblasts and hepatocytes (26, 27). Data from experimental animal infections suggest that *in vivo*, *F. tularensis* is rarely found extracellularly and replicates predominantly within the macrophage (24, 25).

Molecular mechanisms involved in the virulence and the intracellular growth of *Francisella* are not known. Two genes required for the intramacrophage growth of *Francisella novicida,* a closely related bacterium, have been reported (28), but their exact function remains to be identified. There have been a number of reports of components in the outer membrane and LPS and their potential role(s) in inducing an immune response (29-36), but the potential use of these components as therapeutic targets or as diagnostic reagents has not been described. At least one cell-surface polysaccharide antigen (O-antigen) has been isolated by acid hydrolysis of the bacterial lipopolysaccharide (37; 38); this antigen is related to antigens on other bacteria such as, but not limited to, *Pseudomonas aeruginosa* and *Shigella dysenteriae* and probably would not provide an appropriate target for therapeutic or diagnostic reagents. Further, the O-antigen by itself appears not to confer immunity to systemic or aerosol challenge with *F. tularensis* (39).

In this disclosure, new methods for the identification and isolation of novel reagents capable of identifying, or aiding in the identification of, *Francisella* strains, such as, but not limited to, those identified in playing a role in human diseases or those identified as capable of being used as a biological weapon, are disclosed. The reagents may be used in diagnostic assays and kits to diagnose the presence of such *Francisella* strains in a sample suspected of containing a *Francisella* strain. The sample may be obtained from a subject, such as a human or an animal, or may be obtained from an environmental source. In addition the reagents identified may be used as research tools for the study and characterization of *Francisella* strains. In one embodiment, the reagents identified are peptides. In one embodiment, the reagents identified may be used to identify molecular targets and these molecular targets may be used as antigens to provide vaccines useful for protection against *Francisella* strains, such as, but not limited those identified

in playing a role in human diseases or those identified as capable of being used as a biological weapon.

The peptides or other reagents identified by the methods disclosed herein, such as those peptides having the amino acid sequences set forth in SEQ ID NOS. 2, 3, 6, 7, 8, 9, 10, and 11, may be used in kits to determine the presence of a *Francisella* strain in a sample. Such diagnostic kits are well known in the art and will generally be prepared so as to be suitable for determining the presence o of a *Francisella* strain that will bind to the peptides or other reagents identified by the methods disclosed. These diagnostic kits will generally include the reagent, such one or more peptides having the amino acid sequences set forth in SEQ ID NOS. 2, 3, 6, 7, 8, 9, 10, and 11, along with suitable means for detecting the reagents as would be readily understood by one skilled in this art. For example, the means for detecting binding of the reagent to the *Francisella* strain may comprise a detectable label that is linked to the reagent. In one embodiment where the reagent is a peptide, the peptide may be linked to the detectable label. Non-limiting examples of detectable labels may include an enzyme, a chromophore, a flourophore, an affinity tag, an additional peptide sequence or a. radioligand. The detectable label may be detected directly or indirectly. Methods for the detection of such detectable labels are well known in the art. Furthermore, one or more detectable labels may be used in combination or in series.

Such kits can then be used in diagnostic methods to detect the presence of a *Francisella* strain wherein one obtains a sample suspected of having such a *Francisella* strain, such as a sample taken from a subject. The sample may be taken from the subjects, blood, saliva, urine, cerebrospinal fluid, genitourinary tract, tissues, bone, muscle, cartilage, sputum, a mucosal membrane or skin. Reagents are introduced to the sample and allowed to bind to any *Francisella* strain that may be present in the sample, and the reagents are detected. A positive detection would indicate the presence of a *Francisella* strain.

Such kits may also contain a positive control *Francisella* strain, a negative control bacterial strain, and negative control reagents. In the case where the reagents are peptides, the negative controls may be peptides that have a sequence divergent from the sequence of the peptides (such as the peptide disclosed in SEQ ID NO. 4), or that have the same amino acid composition as the peptides, but in a scrambled sequence (such as the peptide disclosed in SEQ ID NO. 5)

One means for identifying such reagents is the technique of phage display. In one method of phage display, random peptide libraries are displayed on the surface of bacteriophage M13 (44-47). Such libraries have been used in a number of different applications, including epitope mapping (47-50), mapping protein-protein contacts (47), and identification of peptide mimics of non-peptide ligands (51-54). Phage display methods do not depend on the protein target being

immunogenic, an advantage over the use of monoclonal antibodies for developing diagnostic reagents. Bioactive peptides can be selected either against immobilized purified targets (55) or against intact cells (56-59). One such library is the Ph.D.-12 library (New England Biolabs), in which 2.7 x 10^9 different random 12-amino-acid peptides have been fused to the amino terminus of the pIII protein, resulting in 3 to 5 copies of a random peptide sequence on the surface of each phage. Other phage display libraries may also be used, as well as other types of libraries.

In the present disclosure, the standard phage display biopanning strategy has been modified to allow for selection of peptides that are capable of binding to *Francisella* strains. The *Francisella* strain to be identified can vary depending on the selection of organisms in the Negative/Positive selection strategy described herein. In one embodiment, the target *Francisella* strain is selected from the group consisting of *F. tularensis* and *F. Novicida,* or sub-species thereof. This strategy has been termed "Negative/Positive" selection, because the first step in each round of selection is a negative selection step, to remove phages that bind to related cell types or organisms. This negative selection is followed by a positive selection that favors binding to the target cell type or organism. A schematic of the steps involved in one embodiment of the Negative/Positive selection step is shown in FIG. 1. The details of the Negative/Positive selection are discussed below.

In one embodiment, the Negative/Positive selection strategy illustrated in FIG. 1 was used to identify peptides that bind to *F. tularensis* LVS and not to *F. philomiragia*. In this embodiment, *F. philomiragia* is used in the negative selection step and is termed the "surrogate strain" and *F. tularensis* LVS is used in the positive selection step and is termed the "target strain". *F. tularensis* LVS and *F. philomiragia* share about 30-40% DNA homology (22). Therefore, some of the differences should represent differences in targets found on the cell surface. The Negative/Positive selection strategy could also detect differences in the amount of individual targets (such as, but not limited to, protein targets) on the surface of the surrogate and target strains, provided that this target was in substantially higher abundance on the target strain than on the surrogate strain. It should be noted that organisms other than *F. tularensis* LVS and *F. philomiragia* can be used as the target and surrogate strains in the Negative/Positive biopanning procedure. For example, *F. tularensis* Shu-4 could be used in place of *F. tularensis* LVS and *F. novicida* could be used in place of *F. philomiragia*. These substitutions are exemplary only and others may be used. In one embodiment the substituted *Francisella* strains are from the same biovar. In an alternate embodiment, the substituted *Francisella* strains are from a different biovar. In addition, more than one surrogate strain or target strain could be used. For example, both *F. novicida* and *F. philomiragia* could be used in the negative selection step. By altering the

organisms used in the negative selection steps, reagents specific for recognition of desired *Francisella* strains and/or reagents specific for only the human pathogenic forms of *Francisella*. Such reagents can be used as important diagnostic tools. The availability of any diagnostic tool for *Francisella* strains will also reduce the chances for confusion of tularemia infection with other diseases with similar symptoms (11, 63).

The percent homology between the *Francisella* strain used in the negative selection step (the surrogate strain) and the *Francisella* strain used in the positive selection step (the target strain) may impact the nature of the reagents identified that bind to the target *Francisella* strain. For example, if the surrogate strain shares a low homology with the target strain, a wider variety of reagents will be identified. Conversely, if the surrogate strain shares a high homology to the target strain, a restricted set of reagents will be identified. The homology may be defined in terms of nucleic acid sequence. In one embodiment, the target strain shares 30-40% homology in its DNA chromosome to the surrogate strain. In an alternate embodiment, the target strain shares 40%-75% homology in its DNA chromosome to the surrogate strain. In yet an alternate embodiment, the target strain shares a 75%-99% homology in its DNA chromosome to the surrogate strain. The percent homology of the target strain to the surrogate strain can be selected by one of skill in the art. Furthermore, the percent homology between the target and surrogate strains can be modulated to identify reagents with desired characteristics.

In addition, the percent homology of the target strain to known human pathogenic forms of the *Francisella* strains can impact the reagents identified. The closer the homology between the target strain and the known human pathogenic forms of the *Francisella* strains, the more likely the reagents identified in the selection strategy will react with pathogenic *Francisella* strains. In one embodiment, the target strain is related to human pathogenic *Francisella* strains such that the reagents identified with the target strain will react with a number of pathogenic *Francisella* strains. Such an increased capacity for recognizing numerous *Francisella* strains can be of value, especially if new pathogenic forms of *Francisella* are required to be detected, as may be the case if *Francisella* were used as a biological weapon. This aspect is applicable to diagnostic testing and treatment, since the treatment for all *Francisella* infections is identical. In one embodiment, the target strain is selected with a percent homology to known human pathogenic *Francisella* strains, such as, but not limited to, *F. tularensis* Shu-4, such that the reagents binding the target strain will react with the human pathogenic forms of *Francisella*. In one embodiment, the target strain shares 30-40% homology in its DNA chromosome to *a* human pathogenic forms of *Francisella*. The percent homology may be determined at the nucleic acid level. In an alternate embodiment, the target strain shares 40-75% homology in its DNA

chromosome to *a* human pathogenic forms of *Francisella*. In a yet another alternate embodiment, the target strain shares 75-95% homology in its DNA chromosome to *a* human pathogenic forms of *Francisella*. The percent homology of the target strain to the human pathogenic forms of *F. tularensis* can be selected by one of skill in the art. Furthermore, the percent homology of the target strain to the human pathogenic forms of *F. tularensis* can be modulated to identify reagents with desired characteristics.

As reagents with specificity for human pathogenic forms of *Francisella* areidentified, the reagents can be used to identify the cell-surface molecule on the pathogenic strains and to identify drug targets specific for the human pathogenic strains.

The affinity and/or avidity of the reagents selected may also be impacted. For example, if the surrogate strain shares low homology with the target strain, then the reagents selected may be of decreased affinity and/or avidity. The number of selection rounds may also impact the affinity and/or avidity of the reagents identified as discussed below.

In summary, by choosing the homology of the surrogate and target strains used in the negative and positive selection steps, the characteristics and specificity of the reagents identified can be modulated. Given the teachings of the present disclosure, one of ordinary skill in the art would be able to choose appropriate surrogate strains and target strains without undue experimentation.

As used in this disclosure, the term "percent homology" of two nucleic acid sequences is determined using the DNA:DNA hybridization method described in reference 22. Other methods of determining the percent homology may be used as is known in the art.

As discussed above, the 12 amino acid Ph.D.-12 Phage Display library was used in one embodiment of the Negative/Positive selection method described herein. Other phage display libraries or other peptide libraries could be used and should be considered within the scope of the present disclosure. In the remainder of this specification, specific reference is made to an embodiment of the methods where peptide sequences are identified as the reagents. In one embodiment of the method disclosed, *F. philomiragia* was used as the surrogate strain and *F. tularensis* LVS was used as the target strains. This embodiment is exemplary only and serves to illustrate the operation of the methods disclosed, and can be altered as discussed above. *F. philomiragia* bacteria were grown in trypticase-soybean broth (supplemented with 0.1% cysteine) to approximately 10^9 (growing in mid-exponential phase). Bacteria were washed at least once with 1X PBS (4°C). 2 x 10^{11} phage particles were added to approximately 10^9 *F. philomiragia* bacteria in 4°C 1X PBS and allowed to bind for 1 hour at 4°C. In alternate embodiments, the binding step can occur at other temperatures, such as, but not limited to, 37°C. In these cases, the

wash steps performed both before and after the binding step are performed at the temperature of the binding step. *Francisella* are resistant to cold, so the 4°C binding step does not affect their viability. After one hour incubation, *F. philomiragia* bacteria were collected by centrifugation and the supernatant added to approximately 10^9 *F. tularensis* LVS bacteria in 4°C 1X PBS. *F. tularensis* LVS bacteria were grown as described above for *F. philomiragia*, and prepared for phage binding as described above. After one hour incubation in 1X PBS at 4°C, *F. tularensis* LVS bacteria were washed three times with 1X PBS (4°C) and then bound phages eluted by treatment with low pH buffer (0.2 M glycine, pH 2.2, at the same temperature used for the binding steps) and neutralized with 1 M Tris/HCl, pH 9.1. This step partially inactivates the bacteria themselves. Any remaining viable *Francisella* bacteria are removed by filtration through a 0.22 micron filter (without detectable loss of phage). Eluted phages were amplified in 20 mL Luria-Bertani medium containing the *E. coli* ER2783 at 37°C (other appropriate *E. coli* strains may also be used). Phage from liquid cultures are obtained by clearing the supernatant twice by centrifugation at 12,000 g for 15 min (each at 4°C), and precipitating with 1/6 vol. of 20% poly(ethylene glycol)-8000/2.5 m NaCl at 4°C overnight. Phage pellets are re-suspended in 1 ml NaCl/Tris (50 mm Tris/HCl, 150 mm NaCl), and precipitated with 20% poly(ethylene glycol)-8000/2.5 m NaCl for 1 hr. Amplified phage are resuspended in 200 ml NaCl/Tris, 0.02% NaN_3, and are used for the subsequent round of biopanning (if desired) to select appropriate peptides.

In the embodiment discussed above, three additional rounds of selection were completed to select for peptides that bound *F. tularensis* LVS preferentially as compared to *F. philomiragia*. Increasing the rounds of selection may decrease the number of candidate phage peptide sequences for analysis and select and/or enrich for those phage having higher specificities and/or higher affinities for *F. tularensis* LVS binding. However, one round of selection may be sufficient. In other instances, additional rounds of selection may be used. One of ordinary skill in the art will be able to determine if additional rounds of selection are required to isolate reagents with desired characteristics. For example, if only reagents with the highest binding affinity are desired, multiple rounds of selection may yield superior results. After 4 rounds of selection using the biopanning strategy above, individual plaques were amplified and phage DNA purified for DNA sequencing, using a modified Sanger sequencing reaction and the sequencing primer: 5' GTATGGGATTTTGCTAAACAAC 3' (SEQ ID NO: 1). From the DNA sequence, the amino acid sequence of the peptide was determined. Two 12mer peptide sequences were isolated after 4 rounds of selection: TSITPWFFLSRP (SEQ ID NO: 2) (phage plaque 4-4-2: found in 4 out of 9 plaques sequenced) and LPISNPLPIKRS (SEQ ID NO: 3) (2 out of 9 plaques sequenced). After 7 rounds of selection, each of these phages were still present in the selected population.

The binding and elution conditions can be selected to favor the isolation of peptides with desired characteristics. These techniques are well known to those of ordinary skill in the art. As an example of such methods, to favor the isolation of phages that express sequences that bind tightly to the target strain, the incubation times for the binding and elution steps can be varied. In one embodiment, to favor the isolation of phages that express sequences that bind tightly to the target strain an initial short (5-10 minutes) elution with 0.2 M glycine (pH 2.2) is used, followed by a second longer elution with the same buffer. Phage that express sequences that do not bind tightly to the target strain will be eluted during the first elution, with phage that express sequence that bind tightly to the target strain being eluted during the second elution. In addition, the first elution could be performed with glycine buffer with a different pH. In one embodiment, the pH of the glycine buffer used in the first elution is pH 3.0 to 6.0. The above example is exemplary only and not meant to restrict other methodologies used to select phages expressing sequences with desired binding characteristics.

In addition to the amino acid sequences disclosed herein in SEQ ID NOS. 2-11, the present disclosure also includes fragments and derivatives of the amino acid sequences disclosed in SEQ IS NOS. 2-11. The fragments are derived from their parent peptides and comprise at least three contiguous amino acids. In an alternate embodiment, the fragments comprise at least 5 contiguous amino acids. A derivative is defined as an amino acid sequence havins conservative amino acid substitutions as compared to the parent sequence. Conservative amino acid substitutions may be viewed as substitutions may be any substitution of amino acids within the same group as shown in Table 1.1 of Biochemistry (Geoffrey Zubay, coordinating author, second edition)

The terms "percentage homology" are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Identity or similarity is evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include,

but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, CLUSTALW, FASTDB

As an alternative to the use of phage display, single-chain antibody libraries (72) can be screened using the "Negative/Positive" selection strategy described. Selection could be done and the selected phage sequences analyzed using approaches like those described elsewhere in this disclosure. In addition, one of the Applicants has constructed a phage display single chain antibody library, taking advantage of the natural single chain antibodies found in camels and llamas. These libraries contain variable regions of 14-19 amino acids and are expressed on the surface of M13 bacteriophage grown in *E. coli*. The "Negative/Positive" selection strategy could be used for these selections as well.

The phage isolated carrying the TSITPWFFLSRP (SEQ ID NO: 2) 12 mer peptide showed preferential binding to *F. tularensis* LVS and *F. novicida* as compared to *F. philomiragia* (FIG. 2). 10^{11} phages were added to cultures of 10^9 *F. tularensis* LVS, *F. novicida*, or *F. philomiragia* cells (mid-log phase growth) in 1X PBS and incubated at 4°C or at 37°C for two hours. After incubation, the cells were washed three times with 1X PBS at room temperature Bound bacteriophage were eluted with 0.2M glycine (pH 2.2) and dilutions were plated onto *E. coli* ER2783 cells as described. Phage titers were in the range of 1×10^5 to 3×10^8 plaques recovered. In FIG. 2, the ratio of binding of the phages to *F. tularensis* LVS and *F. novicida* to *F. philomiragia* were compared. At both binding temperatures, there was greater than a 100 fold binding of phage 4-4-2 expressing peptides having the sequence of SEQ ID NO: 2 to *F. tularensis* LVS and *F. novicida* as compared to *F. philomiragia*. As a negative control, a phage expressing the sequence TGRHTPWPHFMP (OV1) (SEQ ID NO: 4), which was obtained by selection on ovarian cancer cells was used. The OV1 phage showed approximately equal binding to *i*all strains tested. Phage binding tests of the peptide expressing SEQ ID NO: 3 showed no specific binding to *F. tularensis* LVS compared to *F. philomiragia*.

SEQ ID NO: 2 was tested for binding to other bacteria that might be encountered in a clinical laboratory (FIG. 3). The binding of phage expressing peptides having the sequence of SEQ ID NO: 2 to gram-negative *E. coli* DH5α (a strain lacking sex pili and unable to grow M13 phage), gram positive *Staphylococcus aureus* (ATCC 6538), and gram positive *Enterococcus hirae* (ATCC 8043) bacteria were examined. Phage binding experiments were performed as described above at 4^0 C and 37^0 C. The *E. coli, Staphylococcus aureus* and *Enterococcus hirea* strains showed equivalent binding to phage expressing peptides having the sequence of SEQ ID NO: 2 (phage 4-4-2) and SEQ ID NO: 4 (OV1 negative control), all in the range of 10^5-10^6 phage recovered from one ml of bacteria in mid-log phase at an $O.D._{600}$ of 1. This level of binding is

similar to the level observed with gram-negative *F. philomiragia* and 20-100 fold lower than the level of phage binding we see to *F. novicida* and *F. tularensis* LVS. Similar results were also obtained using *Pseudomonas aeruginosa*. These results show that the biopanning strategy described can identify phages expressing peptide sequences that can identify *Francisella* strains, such as, but not limited to, *F. tularensis* LVS and *F. novicida* and related strains, in the presence of bacteria that are routinely encountered in the clinical laboratory setting.

In order to characterize the target to which the peptide of SEQ ID NO: 2 binds, the surface of *F. tularensis* LVS and of *F. philomiragia* was digested with trypsin for 1 hour at 4°C or at 37°C (FIG. 4). Soybean trypsin inhibitor (STI) was then added to inhibit the protease and the cells were washed with 1X PBS 3 times. Phage binding experiments were performed as described above. The 37°C experiment is shown in FIG. 4 and is typical of the results observed. For phage 4-4-2 expressing peptides having the sequence of SEQ ID NO: 2, there was a distinct trypsin sensitivity to binding to *F tularensis* LVS, while there is a small but reproducible increase in binding by this phage to *F. philomiragia* under the same conditions. On both bacteria, the negative control phage expressing peptides having the sequence of SEQ ID NO: 4 showed substantially lower binding and an increase in binding after trypsin treatment, as if trypsin treatment reveals a target to which the phages can bind. As can be seen in FIG. 4 when compared to FIG. 2, after trypsin digestion the level of phage 4-4-2 (expressing SEQ ID NO: 2) binding to *F. tularensis* LVS is 10-20% that seen when no trypsin is used. This strongly suggests that the molecule to which SEQ ID NO: 2 binds is sensitive to trypsin digestion.

In order to verify that the binding observed was specific for the peptide shown in SEQ ID NO: 2, competition experiments were performed (FIG 5.). 1×10^{11} phage expressing peptides having the sequence of SEQ ID NO: 2 (phage 4-4-2) or SEQ ID NO: 4 (OV1 negative control) were mixed with peptides p442 having the sequence of SEQ ID NO: 2 (positive control competing peptide) or p442S having the sequence SLFWTTPIPSFR (SEQ ID NO: 5, scrambled negative control non-competing peptide having the same amino acid composition as the peptide shown in SEQ ID NO: 2). For p442, the peptide was used at concentrations of 0, 150 nM, 1.5 uM and 15 uM; for p442S, the peptide was used at concentrations of 0, 165 nM, 1.6 uM and 16.5 uM. The phage and peptide were added to 1×10^9 *F. tularensis* bacteria and allowed to bind for 1 hour at 4°C in 1x PBS. After binding, bacteria were washed three times with ice cold 1 x PBS. Bound phage were eluted with 0.2 M glycine buffer (pH 2.2) and titered on *E. coli* strain ER2738. As can be seen from FIG. 5, the addition of p442 having the sequence of SEQ ID NO: 2 decreased binding of phage 4-4-2 to *F. novicida* bacteria. The addition of p442S having the sequence of

SEQ ID NO: 5 (negative control) did not inhibit the binding of phage 4-4-2. Similar results were obtained for *F. tularensis* LVS.

The buffers used in the binding and wash steps can also influence the binding characteristics of the reagents described in the present disclosure. The previous examples described above used 1X PBS as the binding buffer and in the various wash steps prior to phage elution. FIG. 6 shows the effects of Tris-Mg buffer, 1X PBS and 0.1X PBS on binding of phage expressing peptides having the sequence of SEQ ID NO: 2 (phage 4-4-2) or SEQ ID NO: 4 (OV1 negative control) to *F. tularensis* LVS, (FT), *F. novicida* (FN) and *F. philomiragia* (FP). 1×10^{11} phage were added to 1×10^{9} bacterial cells as indicated in the various buffers shown. Phage were allowed to incubate for 1 hour at 4^{0}C and were washed 3 times with the same buffer used for phage binding. Bound bacteriophage were eluted with 0.2 M glycine (pH 2.2) and titered on *E. coli* ER2738. The number of phage bound under each condition is shown in FIG. 6. As can be seen, the use of Tris-Mg buffer favored the binding of phage 4-4-2 to *F. tularensis* LVS cells over *F. novicida* and *F. philomiragia*. Using Tris-Mg buffer, phage 4-4-2 showed specificity for the identification of *F. tularensis* LVS. Binding of the OV1 phage to *F. novicida* and *F. philomiragia* was equal to or greater than the binding of phage 4-4-2 to these bacteria. The use of 1X PBS favored the binding of phage 4-4-2 to *F. novicida* over *F. tularensis* LVS and *F. philomiragia*. However, in 1X PBS buffer, phage 4-4-2 showed specificity for the identification of *F. tularensis* LVS and *F. novicida*. Binding of the OV1 phage to *F. philomiragia* was equal to the binding of phage 4-4-2 for this bacterium. Of note, the number of phage 4-4-2 binding *F. tularensis* LVS bacteria was not decreased significantly in the 1X PBS buffer. However, the amount of phage 4-4-2 binding to *F. novicida* bacteria was increased. Neither phage expressing peptides having the sequence of SEQ ID NO: 2 (phage 4-4-2) or SEQ ID NO: 4 (OV1 negative control) bound the *Francisella* strains tested in 0.1X PBS. This data suggests that the components of the wash and binding buffers can influence the binding specificity and selectivity with which the peptide reagents interact with their cellular targets.

The composition of the growth media used to grow the *Francisella* strains may also influence the binding characteristics of the reagents identified in the present disclosure. FIG. 7 shows the effect of trypticase soy broth (TSB) and Chamberlain minimal media on phage binding. TSB is a richer growth medium than Chamberlain. *F. tularensis* LVS (FT), *F. novicida* (FN) and *F. philomiragia* (FP) bacteria were grown in either TSB or Chamberlain minimal medium to mid-log phase. Bacteria were collected by centrifugation and resuspended in 1X PBS at a concentration of 5×10^{9} bacteria/ml. 10^{11} bacteriophage were added to the bacteria in 0.2 ml of 1X PBS at either 4°C or 37°C. After 1 hour at 4°C or 37°C, the bacteria were washed 3 times

with 1X PBS (at the temperature used for binding). Bound bacteriophage were eluted with 0.2 M glycine (pH 2.2, at the temperature used for binding) and titered on *E. coli* strain ER2738. Phage used for binding were 4-4-2 (expressing peptides having the sequence of TSITPWFFLSRP) (SEQ ID NO: 2) or OV1 (expressing peptides having the sequence TGRHTPWPHFMP) (SEQ ID NO: 4) which served as a negative control. As can be seen in FIG. 7, the growth on TSB stimulated binding of phage 4-4-2 on *F. novicida* and *F. tularensis* LVS, although the stimulatory effect observed with *F. tularensis* LVS was not as great. This suggests that the target of the peptide expressed by the 4-4-2 phage can be increased by growth in rich media.

In order to optimize the Negative/Positive selection strategy employed in the present disclosure and to ensure the safety of the selection strategy, several assay parameters were evaluated. First, the sensitivity of *Francisella* strains and the phage to low pH was evaluated. The selection strategy uses a pH 2.2 wash to remove bound phage. After a 15 minute incubation of *F. tularensis* LVS or *F. philomiragia* at 4°C with 0.2 M glycine/HCl buffer (pH 2.2), the bacteria were washed with trypticase soy broth supplemented with 0.1% cysteine (TS-C) and plated on cysteine-heart agar supplemented with 5% defibrinated horse blood (CHA-B) to assess colony formation. Treated *F. philomiragia* cells were unable to form colonies after incubation at low pH. Treated *F. tularensis* LVS cells were partially inactivated by the acidic treatment (70% inactivation). This result provides a level of biosafety to the selection strategy, since the bacteria appear to be significantly inactivated by treatment at low pH. The affect of the low pH on phage titre was also examined. $1x10^6$ phage were incubated with pH 2.2 glycine-HCl buffer for 0, 5, 10, and 20 minutes at 4°C and at 37°C. After incubation, the phage solution was neutralized with 1 M Tris base, pH 9.1, and the phage titer determined. There was no significant decrease in the phage titer after even 20 minutes in acid indicating the phage was not adversely affected by the pH 2.2 wash.

It had been reported that heat inactivation of *Francisella* strains can be accomplished by incubation at 60°C for 10-20 minutes (61). There was a dramatic decrease in viable *Francisella* bacteria recovered after heat treatment, with no viable colonies were recovered after 20 minutes at 60°C. In addition, incubation of M13 phages, such as, but not limited to, those found in the phage display library used in the current disclosure, at 60°C for 1 hour does not significantly reduce the phage titer. An alternative or additional means to inactivate *Francisella* strains is UV inactivation (70). A UV dose of <20 J/m^2 will reduce the viability of *F. novicida* by at least 5 logs within 20 minutes at room temperature.

Independently of the pH and heat inactivation of the bacteria, filtration through a 0.22μm filter was also shown to remove viable *Francisella* bacteria, as determined by plating filtered

bacterial cultures on CHA-B plates and observing colony formation. As a result, the bound bacteriophages can be recovered and the *Francisella* bacteria eliminated in two fast and simple steps.

Finally, the ability of the added phage to lyse the *Francisella* strains used was evaluated. Using a spot assay with dilutions of phage lysate (10^7, 10^9, 10^{11} phages per ml) on lawns of *F. tularensis* LVS and of *F. philomiragia*, it was determined that the phages did not appear to lyse the *Francisella* strains used in the selection assay.

In the present disclosure, *Francisella* strains are grown on cysteine heart agar plates supplemented with 5% defibrinated horse blood (CHA-B) for 48 hours at 37°C, as previously described (69). For binding experiments, tryptic soybean broth supplemented with 0.1% cysteine (TS-C) was inoculated with one colony from a CHA-B plate and incubated overnight at 37°C. Inoculated broth culture are incubated until the bacteria density reaches 10^8 to 10^9 CFU/ml. Stock of bacteria will be stored frozen at -70°C after addition of 10% glycerol.

Determination of Residues Critical for Binding

To further characterize the specificities involved in peptide binding, the amino acid determinants within the peptide sequence of SEQ ID NOS. 2 and 3 that are important for binding were determine using a selection method described by Sidhu and co-workers (71). In this method alanine residues are substituted at each position in a known peptide binding sequence. By careful analysis of the genetic code, this strategy can allow substitution of alanine at any position within the sequence (see Table 1, which is adapted from reference 71). After preparing such a library of alanine substituted phages with the peptide sequences of SEQ ID NOS. 2 and 3, a Negative/Positive selection was performed as described herein and bound phages recovered. Specifically, *F. philomiragia* was used as the surrogate strain and *F. novicida* was used as the target strain. A representative number of phages are sequenced and the frequency with which alanine residues appear at each position within the peptide sequence of SEQ ID NOS. 2 and 3 will be determined. Amino acids positions with a high frequency of alanine residues indicate positions not important in determining binding specificity. Positions with a low frequency of alanine residues indicate positions that are important for binding. In this manner, the critical amino acid residues responsible for binding can be determined.

Once the amino acid positions important to peptide binding are identified, these positions can be substituted to improve the binding characteristics of the peptides, such as, but not limited to, specificity and avidity, of the identified peptides using targeted selection approaches. As an alternative means to improve binding specificity and/or avidity, a new random peptide sequence library can be constructed by incorporating random amino acid sequences on or both sides of the

identified peptide sequence important for binding. The number of residues added is variable and may be as few as one residue or as many as 12. With these additional peptide libraries, additional "Negative/Positive" selection as described above can be performed to identify additional peptide sequences. Additional peptide sequences can be further characterized as described above. In this manner, the specificity and/or avidity of identified peptide sequence can be modulated.

A library of alanine substituted phage sequences were produced where every position of SEQ ID NO. 2 (TSITPWFFLSRP) was substituted for alanine to determine which amino acids in SEQ ID NO. 2 are important for 4-4-2 phage binding. In the case of 4-4-2, this strategy results in a library approximately 260,000 different sequence permutations (6 positions with 2 amino acid choices and 6 positions with 4 possible amino acid choices). A library was prepared (final titer 10^{13} phages per ml or 3.9×10^7 of each possible phage) and analyzed the sequences of 50 plaques to tally the number of substitutions obtained at each position (Table 2, third column). Statistically this 50 plaque sample from the library was not significantly different that predicted, except at positions S2, I3, S10, and P12; the deviation from expectation at these positions may represent sequences that provided some survival disadvantage to the phages during preparation of the library.

Five rounds of Negative/Positive selection were performed as described above with the 4-4-2 alanine-substituted phage library using *F. philomiragia* as the surrogate strain and *F. novicida* as the target strain (Table 2). After selection, the frequency was assessed for each possible amino acid found at each position within the 4-4-2 sequence. Amino acids positions with a high frequency of alanines or other amino acids after selection suggest positions that are not important in determining binding specificity of the peptide. Amino acids positions with a low frequency of alanines or other amino acids after selection suggest positions that are important in determining binding specificity of the peptide. Results from plaques picked after the Negative/Positive selection were analyzed as described (Table 2) and showed that 6 amino acids appear to be important for binding of the 4-4-2 peptide (SEQ ID NO. 2). As can be seen in Table 2, the P at position 5, the W at position 6, the F at position 7, the V at position 8, the L at position 9 and the R at position 11 are important for binding of the 4-4-2 peptide sequence (SEQ ID NO. 2). Therefore the peptide sequence important for binding is PWFVLXR (SEQ ID NO. 6) and PWFLV (SEQ ID NO. 7). In the original 4-4-2 peptide, position 8 was a phenylalanine (F), but analysis of the alanine-substituted library indicates that valine is more often recovered after selection on *F. novicida* (frequency 0.10 for F and 0.88 for V). The serine at position 2 and the isoleucine at position 3 also appear to be selected against. Only one amino acid (the P at position 12) appears at a frequency significantly different from that expected after selection on *F. philomiragia*. When the frequency is analyzed for individual peptide sequences among these 41

individual isolates after selection on *F. novicida*, 20 of isolated peptide sequence displayed the AATTPWFVLSRA peptide sequence (SEQ ID. NO: 8) and 12 of the isolated peptide sequences displayed the TAAAPWFVLARP peptide sequence (SEQ ID. NO: 9). In both SEQ ID NOS. 8 and 9, the core PWFVL (SEQ ID NO. 7) and PWFVLxR (SEQ ID NO. 6) sequences were retained. Specifically, the sequences PWFVLSR (SEQ ID NO. 10) and PWFVLAR (SEQ ID NO. 11) correspond to the identified conserved binding sequences.

The binding of phages AATTPWFVLSRA and TAAAPWFVLARP to *F. novicida* and *F. philomiragia* has been tested and the affinity of binding is similar to that observed with the 4-4-2 peptide sequence (SEQ ID NO. 2).

All references cited herein are incorporated by reference to the extent allowed. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure. The following claim is appended for the purpose of foreign priority only.

PROPHETIC EXAMPLES

Evaluation of Binding Specificity

In order to more carefully evaluate the binding specificity of the peptides identified, fusion proteins could be constructed by incorporating one copy of the identified peptide. In the Negative/Positive selection steps discussed above, the phage expressing the peptides express 3-5 copies of each peptide. In many cases, it is desirable to characterize the binding further in a situation where only one copy of the peptide is present. For such studies, one copy of the identified peptide sequences to be tested are fused to the C-terminus of either enhanced green fluorescent protein (EGFP) or to the amino terminus of β-galactosidase or other marker protein (such as, but not limited to, but not limited to green fluorescent protein, horseradish peroxidase and other marker proteins commonly used in the art). Such constructs will allow a more carefully measurement of binding specificity, to show that binding is peptide-specific, to determine the number of binding sites, and to measure the binding constant. By using fusion proteins to the N-terminus (β-galactosidase) or C-terminus (EGFP), the importance of having a free N- or C-terminus for binding specificity or avidity can be determined.

In one embodiment, candidate peptide sequences identified by the selection strategy above will be fused to the C-terminus of EGFP. Fusion proteins will be constructed to incorporate histidine or other appropriate affinity tags for easy purification of the fusion proteins. PCR may be used to add the identified peptide sequences onto the EGFP gene in an expression plasmid. As

one negative control, a histidine-tagged EGFP construct will be included. As a second set of negative controls, EGFP fusions with the same amino acid composition as the identified peptides but with the amino acids in a different random order (generated using the Genetics Computer Group program SHUFFLE) will be generated (the peptide shown in SEQ ID NO: 5 was generated using this technique). Once amplified by PCR, the DNA product will be digested with restriction endonucleases for cloning into an expression vector. In one embodiment, BglII and HindIII restriction endonucleases for cloning into the pET32a vector (Novagen) are used. Individual clones will be characterized from miniprep DNA (Qiagen) and the DNA sequence determined using the T7-reverse sequencing primer to sequence across the end of the pET32a vector. Correct clones will be transformed into an appropriate host, such as, but not limited to, *E. coli* BL21(DE3) cells, for expression of protein. Protein will be purified by standard methods, such as, but not limited to, by affinity purification on nickel affinity columns (Qiagen).

The purified proteins will be incubated with *F. tularensis* LVS or other appropriate *Francisella* strains to test for binding. Varying amounts of purified EGFP-peptide protein will be mixed in MH broth. After binding for 15 minutes at room temperature, at 37°C, or at 4°C, the bacteria will be washed three times with PBS (pH 7.2) or with MH broth. Binding will be assessed by the relative amount of fluorescent material visible during observation of the bacteria in a fluorescent microscope.

As an alternative, the peptide sequences can be fused to the amino terminus of β-galactosidase and X-gal used as a reagent to detect activity of the bound enzyme, by looking for the conversion of X-gal from a clear solution to a blue color. Using enzymatic methods for detection, the signal can be amplified by modulating the time for development. The fusion proteins would be constructed with histidine or other appropriate affinity tags, to allow easy purification of the fusion protein as discussed above. If necessary, any bound fusion protein can be stripped with mild acid (as done for the selection assays) or whole cells with fusion protein still bound could be used. In these experiments, an X-gal solution is incubated with the protein or cells and the OD_{420} determined. The experiment will be conducted so that the reaction is in the linear range with appropriate standard curves with which to compare the observed β-galactosidase activity. Controls would include β-galactosidase fusions with peptides for scrambled sequences and β-galactosidase protein alone as discussed above.

To demonstrate binding specificity of the identified peptide, competition experiments with a competing peptide will be performed. Competing peptide would be the same sequence as the peptide attached to EGFP or β-galactosidase. For immunofluorescence experiments, varying amounts of the competing peptide would be added with known amounts of the purified EGFP/β-

galactosidase-peptide fusion protein immediately prior to addition to the cells. Negative controls would be no peptide added or the scrambled peptide sequence (same amino acid composition but in a different sequence, as described above).

The binding affinity and the numbers of binding sites for the identified peptides can be determined by Scatchard Analysis as is standard in the art. In one embodiment, purified proteins will be labeled with ^{125}I to a specific activity of 1-2 μCi/μg, with CPM/μg determined by counting in a gamma counter and by Bradford assay. In one embodiment, the marker protein is EGFP. In this embodiment, labeled EGFP, labeled EGFP-peptide fusion proteins and labeled EGFP-scrambled peptide fusion proteins are prepared. Other marker proteins may be used, such as, but not limited to, but not limited to green fluorescent protein, horseradish peroxidase and other marker proteins commonly used in the art. The CPM/μg of protein will be obtained for each ^{125}I-labeled protein. *F. tularensis* LVS and *F. philomiragia* cells, or other appropriate *Francisella* strains, at a known density (such as, but not limited to, 10^8/ml) will be incubated with known amounts of labeled protein at 4°C and at 37°C for one hour in PBS. After binding, the bacteria will be washed with PBS and the bound and free cpm determined by counting in a gamma counter. The binding affinities and the number of binding sites will be determined using Scatchard analysis.

In addition to the above, the purified fusion proteins (EGFP, EGFP-peptide fusion proteins and EGFP-scrambled peptide fusion proteins, or their β-galactosidase counterparts) will be tested with a broad range of gram positive and gram negative bacteria to further characterize binding specificity. Examples of bacteria suitable for such use include, but are not limited to: *Bacillus subtilis, Bacillus anthracis, Listeria monocytogenes, Staphylococcus aureus, Streptococcus pyogenes, Enterococcus hirae, Escherichia coli, Legionella pneumophila* and *Yersinia enterocolitica*. Cultures of each of these bacteria will be incubated with varying amounts of the EGFP- or β-galactosidase-peptide fusion proteins to assess the relative binding of protein by fluorescence or by conversion of X-gal to a blue color as discussed above.

Determining the Molecule(s) to Which the Identified Peptides Bind

In addition to use as diagnostic tools, the peptide reagents identified using the Negative/Positive selection strategy discussed can be used as research tools. The identity of the molecules bound by each identified peptide can be determined. The target of the identified peptides may be either a protein or other cell surface molecules, such as, but not limited to, a complex carbohydrates or lipopolysaccharides. The presence of a protein component as involved in peptide binding can be determined by trypsin digestion, or digestion with other appropriate enzymes. Using the identified peptides, it will be possible to determine what molecules are present on the target *Francisella* strain (for example *F. tularensis* LVS) that are not present on the

Francisella strain used in the negative selection step. Using this approach, it will be possible to identify proteins that may be involved in the pathogenesis of *Francisella* strains. These cell-surface molecules might also provide research tools and specific drug targets that could be useful candidates for new therapeutics and for vaccine development. Additionally, these cell surface markers could provide new and more rapid diagnostics for tularemia.

To characterize cell surface molecules, fusion proteins expressing the identified peptide sequences or the shuffled version of the peptide sequences will be constructed. In one embodiment GST fusion proteins are used although other fusion proteins may also be employed as is known in the art. The PCR strategy to be used will be similar to that described for EGFP and β-galactosidase fusion proteins above. Proteins will be expressed in a suitable host, such as, but not limited to, *E. coli* BL21(DE3), and purified. If GST fusion proteins are used a Glutathione Sepharose 4B column (Amersham Pharmacia Biotech) can be used for purification. The same column can be used for affinity purification of the cell surface molecules, from whole cell extracts of the appropriate *Francisella* strain. Alternatively, the identified peptide sequences or the shuffled version of the peptide sequences can be synthesized with an N- or a C-terminal cysteine, to allow direct coupling to an affinity matrix for subsequent purification.

If the target molecule bound by the identified peptide is a protein, the target will be purified by affinity chromatography to purify it sufficiently to submit for analysis and sequencing by mass spectrometry, or other appropriate means. If the molecule is a complex carbohydrate or lipopolysaccharide, appropriate analysis of complex carbohydrates and polysaccharides will be performed.

Preparation of cell extracts

The target molecules to which the identified peptides bind are expected to reside on the cell surface. Whether the target molecule is a protein, LPS, complex carbohydrate or other molecule, inactivated cell extracts (prepared as described above) can be used to purify the target molecule. Cell extracts are prepared from a suitable *Francisella* strain. In one embodiment *F. novicida* is used. Because the amount of specific binding is high on *F. novicida* and because of the potential to perform insertional mutagenesis in this organism (69) which we will use to confirm the identity of the cell-surface molecule to which phage 4-4-2 binds, we will start purification with this organism.

Analysis of Target Molecules

To determine if the target molecule contains LPS, the methods described by Dreisbach et al (34) will be used. Appropriate *Francisella* cultures are collected by centrifugation, extracted

with methanol and acetone, lyophilized, and then extracted with hot phenol. After treatment with DNAse and RNAse, the LPS will be analyzed for binding by the identified peptides.

To determine if the target molecule is an outer membrane protein, the methods described by Fulop et al (33) will be used. Sonication will be used to disrupt the cells and sodium N-lauroyl sarcosinate will be used to solubilize the outer membrane proteins. SDS-PAGE can be used to analyze the proteins present in this preparation. The outer membrane proteins will be analyzed for binding by the identified peptides.

Several methods may be used to analyze binding of the identified peptide to isolated proteins. The identified peptides may be labeled with ^{125}I, at a tyrosine residue incorporated at the end of the synthesized peptides. The purified cell protein fractions may be purified by size by SDS-PAGE, blotted with nitrocellulose or other membrane, and probed with the radioactive peptides to identify fractions containing binding activity. Alternatively, the EGFP-peptide fusions discussed above may be used to perform co-immunoprecipitation experiments to purify the bound complex in conjunction with anti-GFP antibodies. If the binding constant is not strong, it may be necessary to cross-link the proteins with the bound fusion protein complex prior to immunoprecipitation. As controls, the negative control EGFP alone and/or EGFP-scrambled peptide fusion proteins could be used. Alternatively, appropriate synthetic peptides corresponding to the peptide sequence of the fusion protein could be added to the co-immunoprecipitation reaction reduce the amount of complex recovered in a competition experiment.

The identification of the proteins identified can be carried out by a number of methods well known in the art. One embodiment of an identification method is mass spectroscopy to determine amino acid sequence, followed by analysis of proteomics databases. The protein complexes may be purified by affinity chromatography or co-immunoprecipitation as discussed above. In addition, peptides extracted from 2D gels and other gels can also be analyzed. Briefly, proteins in gel pieces are hydrolyzed *in situ* with trypsin and the resulting peptides analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). The parent proteins are identified by submitting the profile of molecular weights of the tryptic peptides for statistical analysis using the MASCOT program at the matrixscience.com website. Confirmation of a protein's identity is carried out of the Qtof2 using MS-MS analysis. If the cell surface molecule is a protein, the protein can be identified once a mass for the protein is determined. A proteomics database containing the *F. tularensis* Schu-4 genome sequence is available and can be used to predict the masses of all predicted peptides in that genome.

Some of the identified peptides might recognize carbohydrate epitopes on the surface of *F. tularensis* LVS. LPS will be isolated from acetone-dried cells by the procedure described by Dreisbach (34) or by Vinogadov *et al.* (37) which involves hot phenol extraction and precipitation of proteins and nucleic acids with TCA. O-specific polysaccharide will be cleaved off the LPS by heating it in 1% acetic acid for 2.5 hr. The O- polysaccharide will then be isolated by means of gel filtration chromatography on Sephadex G-50. If peptides are found to bind to the O-specific polysaccharide, then its monosaccharide composition and structure will be further studied. Structural characterization of the O-antigen of *F. tularensis* strain 15 revealed that it consisted of a tetrasaccharide repeating unit containing the uncommon amino sugars, N-acetylquinosamine, N-formylquinosamine, and N-acetylgalacturonamide (37). These amino sugars formed glycosidic bonds that were resistant to the usual acid hydrolysis conditions. Despite the inability to obtain free monosaccharides upon acid hydrolysis, Vinogadov *et al.* (37) was able to show that strain 15 O-antigen contained a tetrasaccharide repeating unit using ^{1}H and ^{13}C NMR procedures. Similar approaches will be used as in the analysis of O-antigen

A genetic approach may be used to confirm the identity of the cell surface marker molecule that is specifically bound by identified peptides. Based on the protein sequence obtained from the biochemical purification experiments described above, the corresponding genes will be knocked-out, and the loss of the cell surface marker on mutated bacteria will be confirmed by the absence of binding by specific peptides to *F. novicida* or other *Francisella* strain. This approach will be used when the marker has been identified as a protein and is likely to be encoded by a single gene.

The main problem with performing knock-out experiments in *F. tularensis* LVS is that no system to generate allelic replacement mutants have been described for this microorganism. However, *F. tularensis* may be used if desired. Allelic replacement has been successfully achieved in *F. novicida* which is very closely related to *F. tularensis* (69, 70). *F. tularensis* and *F. novicida* are virtually identical by 16S rDNA sequencing and by DNA hybridization and, although less virulent, *F. novicida* is still able to cause a tularemia-like illness in humans in rare cases (21, 22). Based on these observations, it has been suggested to consider *F. novicida* as a subspecies of *F. tularensis*. In addition, as shown in FIG. 2, *F. novicida* was shown to strongly bind phage expressing SEQ ID NO: 2. Therefore, *F. novicida* is a legitimate surrogate for *F. tularensis* in our knock-out experiments.

Briefly, the presence of a target molecule identified by a peptide as described in this disclosure will be confirmed in *F. novicida* by Southern blot analysis. DNA probes will be identified based on the known sequence from *F. tularensis* Schu-4 or by creating DNA probes specific for the amino acid sequences of the purified protein product encoded by this gene. Once

identified, the gene will be then isolated from *F. novicida* genomic DNA and cloned into an appropriate vector, such as, but not limited to, pBlueScript KMII. Either a PCR amplification strategy (if the homology between *F. novicida* and *F. tularensis* Schu4 is high in this region) or a shotgun cloning strategy followed by identification of desired clone with DNA probes) can be used for this purpose. The resulting construct will then be subjected to *in vitro* transposon mutagenesis, using the EZ::TN<R6Kγori/KAN-2> Insertion kit from Epicentre Technologies (Madison, WI). This kit can be used to randomly insert the <R6Kγori/KAN-2> transposon into target gene cloned into the plasmid DNA *in vitro*, using kanamycin as a resistance selection marker. An aliquot of the insertion reaction will then be transformed in *E. coli* and transformed cells will be plated on LB plates containing 50μg/ml kanamycin. Transposon insertion clones can be sequenced from each end of the insert using forward and reverse transposon-specific primers, and only the clones carrying an insertion within the gene of interest will be selected.

The knock-out experiment will be performed as previously described (69) This approach is based on the fact that it is possible to transform *F. novicida* with plasmids exhibiting narrow host range (i.e., unable to replicate in *F. novicida*) carrying *Francisella* chromosomal DNA fragments and a kanamycin resistance marker (28, 69, 70). This observation has been exploited to generate knock-out mutants by homologous recombination in *F. novicida*. Constructs carrying the gene of interest interrupted by EZ::TN transposon will be introduced into *F. novicida* by chemical transformation as previously described (70) and cells will be plated onto CHA-B plates containing kanamycin. Kanamycin-resistant transformants will be isolated after 24 to 48h incubation at 37°C. Allelic exchange between the wild-type gene and the mutated gene will be confirmed by Southern blot analysis of chromosomal DNA from each kan^+ recombinant, using either the cloned gene itself or the EZ::TN transposon as a probe.

Mutants inactivated for a particular cell surface marker will then be tested for their binding of the corresponding peptide as described above. For example, in the case of phage 4-4-2, we expect absence of specific binding by the phage as a consequence of the loss of the marker. The binding potential of mutant *F. novicida* knock-outs can be determined as described above.

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Amino acid	Shotgun codon	Possible substitutions	Expected frequency of the correct amino acid
F	K (C/T) T	A/F/S/V	0.25
I	(A/G) (T/C) T	A/I/T/V	0.25
L	(G/C) (T/C) T	A/L/V/P	0.25
P	(G/C) C A	A/P	0.5
R	(G/C) (G/C) T	A/R/G/P	0.25
S	(G/T) C C	A/S	0.5
T	(A/G) C T	A/T	0.5
W	(G/T) (G/C) G	A/W/G/S	0.25

Table 1: Substitutions that allow random substitution of alanine at any position in an amino acid sequence (adapted from 71). These amino acid codons will be initially introduced into the peptide sequence in phage plaque 4-4-2 (TSITPWFFLSRP), for selection of new peptide sequences that might show more avidity and/or specificity for binding to *F. tularensis* LVS over *F. philomiragia*.

		Observed frequency		
position	expected frequency	Unselected N = 50	Selected on *F. novicida* N = 41	Selected on *F. philomiragia* N = 9
T	0.5	0.48	0.44	0.56
S	0.5	**0.70 ****	**0.20 ****	0.67
I	0.25	**0.12 ***	**0.00***	0.22
T	0.5	0.6	0.56	0.33
P	0.5	0.46	**0.93 ****	0.56
W	0.25	0.22	**0.88 ****	0.33
F	0.25	0.20	**0.93 ****	0.11
F	0.25	0.14	**0.10 (V = 0.88 **)**	0.22
L	0.25	0.14	**0.98 ****	0.11
S	0.5	**0.32 ***	0.54	0.67
R	0.25	0.18	**0.85 ****	0.22
P	0.5	**0.74 ****	0.49	**1.0 ****

Table 2: Statistical analysis of unselected and selected 4-4-2 libraries substituted with alanines. N = number of individual plaques whose sequences were analyzed. P-values were assigned by analyzing the deviation of the observed frequency for each amino acid position from the expected frequency, using a bionomial distribution (at each position, the frequency of the correct amino acid, with all other amino acids at that position considered not correct). Frequencies followed by * are significantly different from expectation ($p<0.05$) and those with ** are highly significantly different ($p<0.01$). In the library selection on *F. novicida*, if the data is calculated to allow V at position 8, then the frequency at that position also becomes significantly different from the expected frequency.